In Vivo Studies of Magnetic Nanoparticles-Folic Acid (FA) Functionalized for Breast Cancer Targeting Drug Carrier

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In Vivo Studies of Magnetic Nanoparticles-Folic Acid (FA) Functionalized for Breast Cancer Targeting Drug Carrier

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Abstract. The number of cancer patients in the world is constantly increasing and it is considered as one of the deadliest among other diseases. There are urgent needs of effective and efficient medicine for late-stage cancer since none of current treatment has promising outcome especially related chemotherapy's side effects. Magnetic nanoparticles with its ability to be modified, coated and functionalized is a candidate for drug delivery's targeting carrier. This research goal was proposing a novel drug carrier to specifically target cancer cells without any significant remnant in other tissues. Our method begun with MNP coating using bovine serum albumin then functionalized with folic acid as targeting agent. Multiple evaluations towards functionalized MNP includes: MTT Assay for its toxicity, in vivo test for targeting capability assessment, and histopathology test for host's cellular response. The results showed that the magnetic nanoparticles-FA functionalized was non-toxic, no significant cellular damage, and it had a promising result to be used for further cancer targeting study.

INTRODUCTION

In 2012, the number of cancer sufferers was approximately 14 million people and fatalities' number was more than 8.2 million [1]. Indonesia, with dire situation in cancer treatment, needs affordable yet effective novel method. Further, economic issues increased the challenge to develop an efficacious and effective medicine. In 2012 only, cancer sufferers' number in Indonesia was increased 347,000 people with 1.4% of prevalence. In fact, current available method such as surgery and the old-fashioned chemotherapy often failed to cure the cancer [2]. Moreover, traditional chemotherapy may be seditious to a lot of organ damage in most of the cases [3]. Magnetic nanoparticles can be done to surmount this problem through their capability to be controlled and modified as targeting carrier for drugs. They will facilitate toxic chemotherapy drug to accurately reach and diminish cancerous cells without damaging any healthy tissues. MNP also has lower synthesis cost compared to the others nanoparticles and magnetic properties for active control through magnetic field. However, magnetic nanoparticles are hydrophobic material and toxic in a high concentration, so it requires appropriate coating to turn it into hydrophilic and non-toxic [4].

Magnetic nanoparticles (MNP) are mainly consisting Fe_2O_3 and Fe_3O_4 including alpha, beta, and gamma Fe_2O_3. Both of them has paramagnetic property but they will become superparamagnetic in nano-scale size. The superparamagnetic property gives MNP capability to be controlled by permanent magnet without any magnetization remnant. In addition, MNP have very large surface area and high affinity to various type of protein especially the one with hydrophile-hydrophobic sides which reduce coating complexity [5].

One particular method to synthesize MNP is co-precipitation method. It is chosen due to its simplicity in size controls and all reaction waste are water soluble which is beneficial during washing process. MNP may be coated using bovine serum albumin (BSA) to reduce its toxicity and increase its stability when suspended in the body fluid. BSA also has the capability to be bonded with drugs especially peptide-based and protein-based drugs since it has

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excess peptide bonding. The folic acid is used to act as a targeting agent since cancerous cells tend to capture folate to maintain its metabolism process [6,7]. Coating process involves the carboxyl and hydroxyl cluster activator such as 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-Hydroxysuccinimide (NHS), and ultrasound bath. In order to ensure materials' safety, magnetic nanoparticles should be evaluated using MTT Assay to check its toxicity against living cells and FTIR to ensure the coating has been appropriately done. Vibrating sample magnetometer is used to assess the magnetic properties before and after coating process.

Particle size is a main concern for drug carrier to be applied since there is limitation in size of membrane leaking in cancerous cells (100-400 nm). So, to assure that particles can penetrate the cells, it's size must be smaller than 100 nm after all coatings and functionalization. It is all due to the fact that coatings may increase particle size significantly and improper coatings may cause flocculation [8]. MNP are tested twice to guarantee its particle size and geometry; particle size analyzer (PSA) to observe before and after diameter, and scanning electron microscope (SEM) to visually observe the shape, the presence of coagulation, and actual particle size.

The evaluation of targeting process is measured through liver and the cancerous tissue's iron (Fe) concentration using ICP-AES [9,10]. The tissue samples are also observed for its histopathology condition such as inflammation, membrane rupture, or diminishing nucleus.

Previous research has been conducted to analyze the magnetic and chemical properties of FA-BSA-MNP along with its toxicity. However, none of those has been injected to animal and measured for its accuracy. This project aims for finding the accuracy of FA-BSA-MNP to target cancer cells in animal model.

MATERIALS AND METHOD

Materials used were pure $FeCl_2$ (99.8%) from Merck, pure $FeCl_3$ (99%) from SAP Chemicals, Phosphate buffer saline (PBS), Folic acid (FA), EDC and NHS from Sigma Aldrich, BSA from Thermo-Fisher Scientific, dimethyl formamide (DMF) from Merck, HCl and H_2O_2 from SAP Chemical, and buffer formalin 10%.

Magnetic Nanoparticles (MNP) Synthesis

$FeCl_2$ and $FeCl_3$ were prepared with 2:1 molar ratio, then both of them were diluted in deoxygenized water 70°C with continuous stirring for 30 minutes. The resulting solution was red-brown colored and clear. Before reacted further, the solution needed to be filtered using multiple layer paper filter (10μm) to eliminate any large sized impurities. Then, the solution was dripped using NH_4OH 25% with 1500 rpm continuous stirring and maintained at 70°C. The dripping process was stopped when the pH reached 9. The solution was turned into an opaque black. It washed for several times with purified water and assisted with permanent neodymium magnet (11000 Gauss) until the pH lowered to 7. Washing process eliminated remaining NH_4OH and any water-soluble impurities. Final suspension was expected to be less concentrated, so pure water was added until it reached 50 ppm concentration [11].

Coating and Functionalization of MNP

Part of 50 ppm MNP suspension was diluted until 20 ppm while BSA, EDC, and NHS were diluted in PBS with ratio of BSA and MNP concentration 1:40 (BSA concentration 800 ppm). The MNP and the BSA solution was mixed then stirred using mechanical stirrer inside an ultrasound bath for 2 hours. The coated-MNP suspension was stored in cold and dry room to prevent albumin damage. Folic acid solution was prepared by mixing the 5mg folic acid with 42 mg EDC and 60 mg NHS diluted in PBS and DMF (PBS: DMF 1:1 v/v) using mechanical stirrer thoroughly. Coated-MNP suspension then was mixed with FA solution inside an ultrasound bath and under continuous stirring for 2 hours. The final result was FA-BSA- MNP suspension. However, excess BSA and FA should be obliterated by using 15000-rpm centrifuge. The final precipitate was collected and suspended with PBS and stored in the refrigerator [12,13].

FA-BSA-MNP Characterization

The magnetic nanoparticle must be verified that it only consisted Fe_2O_3 and Fe_3O_4 without any impurities including pure Fe. First, X-ray Diffractometer (XRD) was used to understand its composition and check its crystallinity. Before the test, the MNP must be dried using oven at 50°C for 24 hours, lower temperature was preferred to avoid coagulation due to abrupt change in Brownian motion. Second, FA-BSA-MNP and BSA-MNP were characterized using Fourier Transform Infrared Spectroscopy (FTIR) to observe the bonding between them and compare for both characteristics.

Since appropriate particles' size and morphology were essential for drug delivery, Particle Size Analyzer (PSA) and Scanning Electron Microscope (SEM) were compulsory test for both of the FA-BSA-MNP's and MNP's. Another test for the particles, before it went to further step was MTT Assay to measure the particles toxicity against living cells. The cells used were human hepatocytes and multiple replication was applied assure the result [14].

In Vivo Application of FA-BSA-MNP

(The ethical clearance for in vivo had been approved by the Faculty of Veterinary Airlangga University). In vivo studies were done to understand the capability of FA-BSA-MNP to target the cancerous cells in a living organism and host response against the MNP. Two tissues were measured, first was the cancerous tissue, the other was liver. The subjects used were 7 Rats (Rattus norvegicus) Wistar strain which was divided into 4 groups. All of the rats were cancer induced using 300 ppm benzo[a]pyrene dissolved in olive oil. The induction process was done by injecting the 0,5mL dissolved benzo[a]pyrene subcutaneously over the mammal glands, every 2 days for 1 month and then they were left for 3 months [15]. After the cancerous lump appeared, 6 rats were injected with 1mL FA-BSA-MNP intravenously through tail vein. Control group of rats, which has no MNP injection, were dissected on the same day as the injection process. The other 6 rats which had been injected by FA-BSA-MNP, are separated into 3 groups. First group (A Group) were operated after 1-hour post injection, second group (B Group) were operated after 4 hours post injection, and the last (C Group) were operated after 8 hours post injection. Their liver and cancerous tissue from all groups were collected and stored in buffer formalin.

Histopathology Test

All of the cancerous tissue and the liver were cut into two, one was used for histopathology test and the other was used for Fe concentration measurement. Histopathology test was used to visually observe the particles' effect on living cells inside the organism body. The organs were sliced, put on the glass and observed under the optical microscope.

Fe Concentration Analysis

This test was done using ICP-AES which had the ability to measure metal concentration using plasma. All of the organs were diluted in the mixture solution of 37% HCl and 30% H_2O_2. Finally, the solution for each sample were tested for their iron (Fe) concentration.

Analytical Method

The main test for this research was in vivo studies where the capability of FA-BSA-MNP was tested to accurately target cancer. It was hypothesized that FA-BSA-MNP will accurately reach the cancerous cells without any significant remains and damage in the liver's tissue. All data will be compared qualitatively due to insufficient data for minimum statistical power. Other tests within this project were done to confirm FA-BSA-MNP safety and properties before it can be safely administered to animals and provide reliable results.

RESULT AND DISCUSSION

Synthesis Result

The synthesis of magnetic nanoparticles from pure $FeCl_2$-$FeCl_3$ gave an opaque black jet suspension. The suspension container then was left for 4 hours on the permanent magnet to precipitate the nanoparticles and the dispersing liquid was removed. MNP then was washed using pure water, and dispersed in PBS to maintain the pH and eliminate excess reactant. The MNP suspension before and after washing process can be seen in figure 1.

(a) (b)

FIGURE 1. a) MNP after synthesis; b) MNP after separated and washed

Coating and Functionalization of FA-BSA-MNP

The MNP's appearance after being diluted to 20 ppm was clear yellow-brown, tea like color which can be seen in figure 2a. After coating process, it had lower concentration and higher stability. It did not settle for more than 2 days which indicated desired stability had been achieved and MNP was ready to be injected into blood stream. Prior the functionalization of BSA-MNP, FA solution must be ensured that all ingredients had diluted perfectly and had no clumps. After functionalization, the BSA-MNP become FA-BSA-MNP which has considerably clear appearance with slightly yellowish color. After centrifuged, the FA-BSA MNP precipitated at the bottom then the filtrate was removed, changed with the PBS, and finally the suspension was stored at cool temperature. The result can be seen in figure 2b

(a) (b)

FIGURE 2. a) BSA-MNP b) FA-BSA-MNP after centrifuged

Characterization of FA-BSA-MNP

XRD and PSA Tests of MNP

The XRD results showed that the MNP owned the characteristics of iron oxide peaks. The main peaks were 30.46°, 35.66°, 57.52°, and 62,97° which represented the peaks of Fe_2O_3 and Fe_3O_4. The average crystallite size, measured by using the FWHM of the dominant peaks, was 27 nm. All of the Fe_2O_3 and Fe_3O_4's peaks are matched with the result which meant no impurity found in the sample, the particle composition was 64% Fe_2O_3 and 36% Fe_3O_4. The XRD peak graphs can be seen in figure 3. Other smaller peak within the samples was regarded as noise due to small size of the particle and they were not solid massive particles. The size measurement using the dynamic light scattering resulted particles' size before coating was 67.19 and 71.89 (first and second measurement). There was minor discrepancy between to measurement due to Brownian motion within the suspension. The particle size's distribution curve had single peak which indicated homogenized particle. The PSA distribution graphs can be seen in figure 4. According to satisfying XRD and PSA results, further testing such as FTIR, toxicity, and in vivo test can be done. Finally, PSA test was done after coating to confirm no coagulation and flocculation present. The result was 105 nm and homogenic in size.

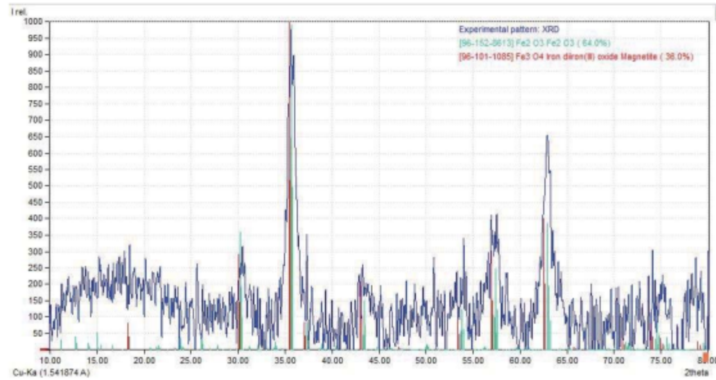

FIGURE 3. XRD result chart, all peaks were matched with Fe2O3 and Fe3O4 peaks

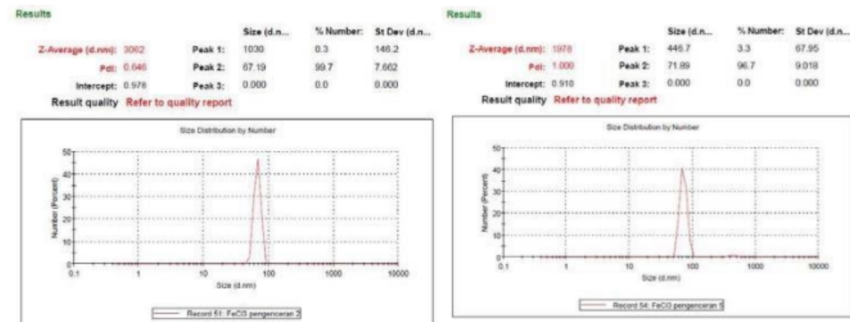

FIGURE 4. PSA size distribution chart a) first trial and b) second trial

FTIR Test of Coated and Functionalized MNP

Second test, was FTIR to verify whether FA and BSA had been bonded properly with the magnetic nanoparticles. Centrifuged the MNP was expected to avoid bias in FTIR readings since it will measure the samples in a suspension form while the BSA-MNP was water soluble. The FTIR results of BSA-MNP showed that there was a slope between the wavenumber 3200cm^{-1} and 1630cm^{-1} which represented the group of -NH and -C=O consecutively. It meant that the BSA had been linked to the MNP via the activated carboxyl group of BSA, since there were no excess BSA in the medium. The FTIR graphs can be seen in figure 5a. The FTIR results of FA-BSA-MNP showed that the slope of -C=O was shallower compared to the BSA-MNP and there was a new peak for -CH which located at wavenumber 1390cm^{-1}. The -CH group was the result of the -COO group activation and it could be implied that the FA had been linked to the BSA through -COO bonding. Other smaller peaks were considered a noise or excess EDC and NHS. The graphs can be seen in figure 5b.

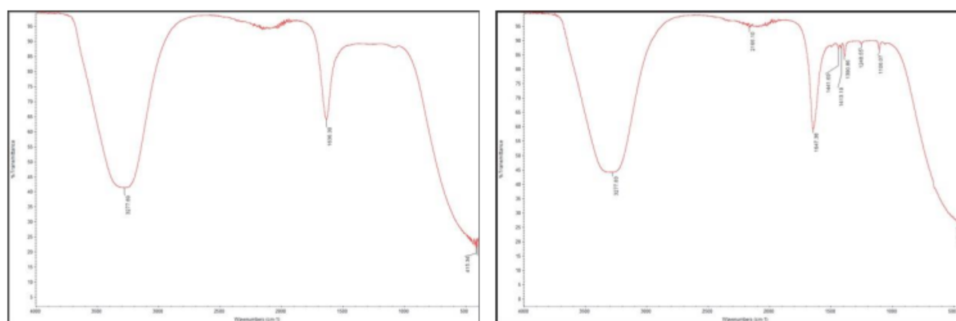

FIGURE 5. FTIR chart a) BSA-MNP and b) FA-BSA-MNP

Morphology Analysis of MNP using SEM

The test was done to both MNP and BSA-MNP, but the FA-BSA-MNP was not tested because the FA did not have the potential to significantly change the geometry. SEM results showed spherical particle shape and several coagulated particles. The sized seen in SEM was similar as the PSA results (60-70 nm) but they tended to attached to adjacent particle. The tendency may be caused by the heat caused by the SEM. The particles' shape was consistent as the prediction and homogenous which were predicted to be able to penetrate cell's wall. The SEM image can be seen in figure 6.

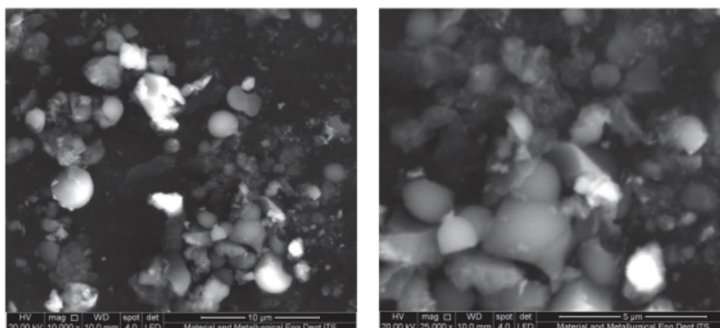

FIGURE 6. SEM image a) 5000x magnification and b) 25000x magnification. All images delineated a spherical particle shape and multiple aggregation occurred during the test

Cytotoxicity Test

This test which was required in advance of in vivo studies, was done by using the hepatocyte and its reaction with MTT salt. The cells viability was calculated by comparing light intensity at wavelength 490 nm for sample well and control well. The result showed that the BSA-MNP and the BSA only sample had non-toxic properties and considerably similar viability. Both of them had no notable effects on the cells. It was proven that the BSA had eliminated the MNP toxicity and thoroughly coated the MNP. The FA did not undergo toxicity test, since the FA also found in the physiological cellular metabolism. Albumin from BSA was a physiological substance which physiologically produced by the liver, this explained the reason of its capacity to obliterate the toxicity. It can be concluded that FA-BSA -MNP was safe as drug carrier for in vivo test. The viability comparison graphs can be seen in figure 7.

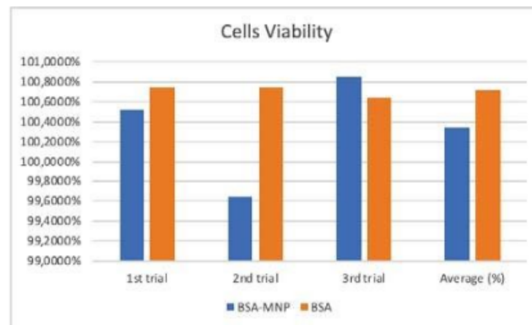

FIGURE 7. Cytotoxicity of BSA-MNP suspension and BSA solution. No significant difference between them indicated BSA had eliminated MNP toxicity

Breast Cancer Induction on Rats

The induction took place for more than 3 months when lump just started to grow. After the lumps were in sufficient size, FA-BSA-MNP were injected via the tail vein while the rats were anesthetized under the effects of Ketamine Sulfate. Once the rats were operated, the lumps were found from both benzo[a]pyrene accumulation and the abnormal tissue growth. The injection process, operation process, and tissue can be seen in figure 8.

FIGURE 8. a) Benzo[a]pyrene injection, b) the lumps, c) FA-BSA-MNP injection, d) operation, e) tissue around mammary gland

Histopathology Test

The general readings represented all of the neoplasia has the cancerous like-tissue with abnormal cells nuclei and cells shape. There were also some sinusoid areas widening around the central vein of the liver which indicating that some of the benzo[a]pyrene also went to the liver. In the A Group, the number of atypical cells was more compared to the control. In the B group, there were trivial increase of erythrocyte number compared to the control groups. In the C group, the sample has more invasive neoplasia, where they had invaded the mamma cells. Among those groups, there was insignificant difference between the FA-BSA-MNP injected groups and the control groups, most of the differences were the result of variative individual response against benzo[a]pyrene. (The significance and the result were mentioned in a qualitative way by the Laboratory of Pathology Universitas Airlangga). The tissue microscopic image can be seen in figure 9.

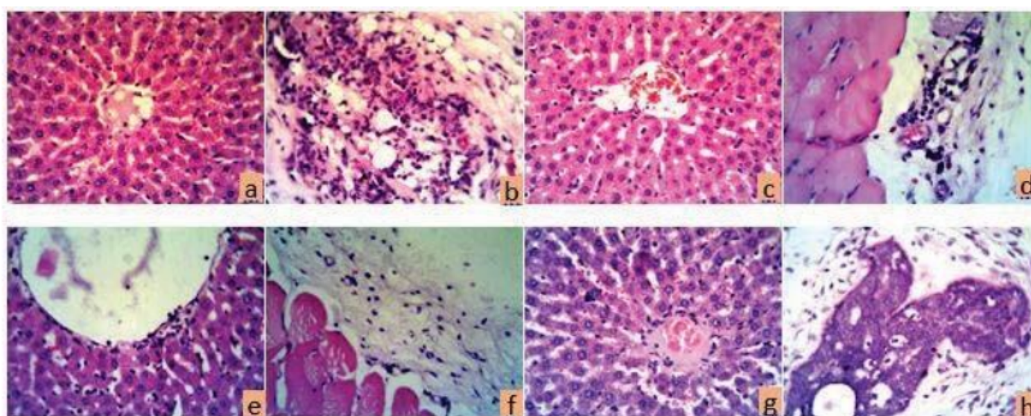

FIGURE 9. Microscopic view of the Rats' a) control group liver, b) control group mamma, c) A Group liver, d) A Group mamma, e) B group liver, f) B group mamma, g) C group liver, h) C group mamma

Fe Concentration Test using ICP-AES

The test was conducted after all organs had been diluted. There was no significant difference between control and injected groups at the liver. There was only one surge on one of the C groups that may be caused by the individual response. Over all as seen in figure 10, there were no concentration increase of iron (Fe) in the liver samples. In the lump or cancerous tissue, the increase was not significant after one-hour post injection. The surge was appeared on 4-hour post injection group. The Fe concentration was 5 times higher compared to the control. It happened for all of the rats in the B group. The C group, had lower concentration of Fe, since after 8 hours the blood may have detoxicate the nanoparticles. Even though the result indicated the MNP directed to cancerous tissue, the iron concentration in the liver was higher compared to cancerous tissue. It was suspected that there were large numbers of hemoglobin from blood cells resided in the liver contained iron. In spite of this fact, high iron level in the liver had no correlation with MNP injection, since the level within treatment group was similar as the control. The results provided early indicator that the nanoparticles possessed the ability to target and accumulated only in the cancer cells.

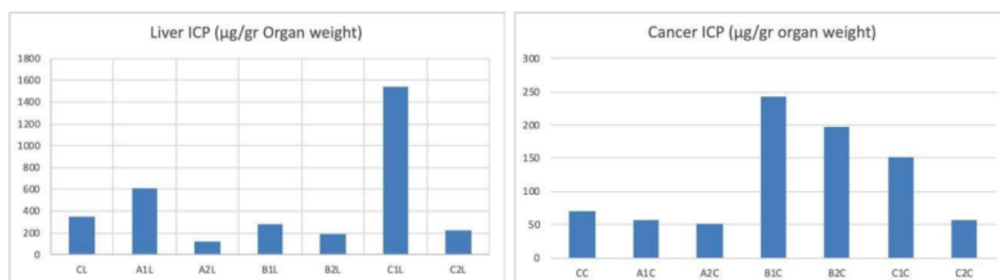

FIGURE 10. ICP bar chart for Fe concentration across different time period in liver and cancerous mammary glands

CONCLUSION

FA-BSA-MNP has the potential to be used as targeting agent. Initial research in animal setting indicated accurate targeting capability and has been proven to be safe for surrounding tissue and cells. Further study with larger sample size and different animal such as primates needs to be conducted to further study its efficacy and accuracy. Then, after the targeting accuracy test, drug loading and release study including in vitro and in vivo, may also be conducted to observe BSA capacity before clinical trials.

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